DOCKET NO: 263270US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	:
STEPHANE MIRAS, ET AL.	: EXAMINER: HAMIDINIA
SERIAL NO: 10/517,309	:
FILED: AUGUST 3, 2005	: GROUP ART UNIT: 1653
FOR: PLASTIDIAL TARGETING PEPTIDE	:

DECLARATION UNDER 37 C.F.R. 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

I, Norbert Rolland hereby declare:

1. I am a named inventor of the above-identified application and am familiar with the specification of the above-identified patent application.

2. The following observations and experiments were carried out by me or under my direct supervision and control.

3. The data shown below demonstrate that the sequence of 94 residues, corresponding to N—terminal amino acids 6—100 of both spinach and Arabidopsis proteins, is sufficient to catalyze the importation; the 223 C-terminal residues (101- 323) of these two proteins are therefore not essential to the importation.

4. A construct encoding a truncated form (amino—acids 6-100) of this 1E41 spinach protein (identified in the application as SEQ ID NO:1), fused to GFP, was expressed in tobacco cells.

5. In the 6-100 region, the spinach 1E41 protein shares 70.0% identity and 82.0% similarity with the same region of the Arabidopsis 1E41 protein.

6. Construction of the expression vectors:

The plasmid [35Ω-sGFP(S65T)] used for these constructs, which comprises the sequence encoding GFP under the control of the 35S promoter, and also the plasmid [35Ω-TP-sGFP(S65T)], which comprises the sequence encoding the targeting peptide (TP) of the ribulose-1,5-bisphosphate carboxylase small subunit, fused to the sequence encoding GFP, were previously described by CHIU et al. (Curr. Biol., 6, 325- 330, 1996).

The sequence encoding the amino-acids 6-100 of the spinach 1E41 protein is amplified by PCR using the following two primers:

SalI-N-ter GCTGTCGACATGCATGCGATTCAATATTCTGGC

NcoI-C-ter ACCCATGGTAGCATGACTAAGCACGGCCAC

which introduce, respectively, a SalI site and an NcoI site (underlined).

The PCR product is cloned, blunt-ended, into the vector pBLUESCRIPT KS (STRATAGENE) . The SalI-NcoI fragment cleaved from the plasmid thus obtained is inserted into the plasmid 35Ω-sGFP(S65T) digested beforehand with SalI-NcoI, in order to create the vector 35Ω-(6-100)SoIE41-sGFP(S65T), comprising the region coding for the amino-acids 6-100 of the spinach 1E41 protein, fused to GFP.

The entire expression cassette of the (35Ω-(6-100)SoIE4l-sGFP(565T))plasmid (containing the 35Q promoter region, the region coding for the amino-acids 6-100 of the spinach 1E41 protein fused to GFP, and the Nos terminator region) was then transferred as an EcoRI/HindIII fragment into a binary vector (pEL103-(6-100)SoIE4l-sGFP(565T)) for agroinfection of plant leaves.

A similar construct was obtained for the Arabidopsis 6-100 region fused to GFP. The entire expression cassette of the (35Ω—(6—100)AtIE41—sGFP(S65T)) plasmid (containing

the 35Ω promoter region, the region coding for the amino-acids 6-100 of the Arabidopsis 1E41 protein fused to GFP, and the Nos terminator region) was also transferred as an EcoRl/HindIII fragment into a binary vector (pEL1O3-(6- 100)AtIE41-sGFP(S65T)) for agroinfection of plant leaves.

As controls, binary vectors (pEL103-sGFP(S65T) and pEL103-TP-sGFP(S65T)) for agroinfection of plant leaves were also derived (transfer of the EcoRl/Hindlil fragments) from the plasmid [35Ω-sGFP(S65T)], which comprises the sequence encoding GFP under the control of the 35S promoter (negative control), and also the plasmid [35Ω-TP-sGFP(S65T)], which comprises the sequence encoding the targeting peptide (TP) of the ribulose-1,5-bisphosphate carboxylase small subunit, fused to the sequence encoding GFP (positive control)

4. Agroinfiltration of plant leaves

The protocol used for the agroinfiltration of plant leaves was previously described by LAVY et al. (The Plant Cell, Vol. 14, 2431—2450, 2002)

5. **Fluorescence microscopy**

The location of the GFP and of the GFP-fusion peptides in analyzed in the transformed cells by fluorescence microscopy using a ZEISS AXIOPLAN 2 fluorescence microscope and a digital CCD camera (HAMAMATSU) . The sets of filters used are: Zeiss filterset 13, 488013—0000 (exciter BP 470/20, beam splitter FT 493, emitter BP 505—530), and Zeiss filter set 15, 488015—0000 (exciter BP 546/12, beam splitter FT 580, emitter LP 590) for the GFP and the chlorophyll autofluorescence, respectively.

Under these conditions, the presence of chlorophyll (specifically located in the chloroplasts) and the location of the GFP in the cell are visualized by virtue of an intense fluorescence.

The results are shown on the enclosed figure where A: GFP fluorescence; B: chlorophyll

autofluorescence; C: superposition of A and B.

In the tobacco cells transformed with the constructs GFP, the GFP fluorescence appears to be diffuse and located in the cytosol and the nucleus; no co—localization with chlorophyll is observed.

In the tobacco cells transformed with the spinach and Arabidopsis constructs (6-100)1E41 fused to GFP, and also with the positive control for plastid localization TP-GFP, a co—localization is, on the other hand observed in the chloroplasts, between the GFP fluorescence and the chlorophyll autofluorescence.

These experiments show that the targeting is effective for both spinach and Arabidopsis constructs (6- 100)1E41 fused to GFP.

6. All the results above show that the sequence of 94 residues, corresponding to N—terminal amino acids 6—100 of both spinach and Arabidopsis proteins, is sufficient to catalyze the importation; the 223 C-terminal residues (101- 323) of these two proteins are therefore not essential to the importation. Moveover, these data show that although the region 6-100 of spinach IE41 has only about 70% homology with the region 6-100 of Arabidopsis IE41, they have the same properties of intraplastidial targeting. Accordingly, in my view these two proteins as described in the application, e.g., on page 22 and the Sequence Listing are represenative of the types of sequences being claimed in the application.

7. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believe to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under

Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

_____

Norbert Rolland

_____

Date